FEBS Letters 581 (2007) 1457–1463

# Identification of the *TPO1* gene in yeast, and its human orthologue TETRAN, which cause resistance to NSAIDs

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Received 19 February 2007; accepted 1 March 2007

Available online 8 March 2007

Edited by Horst Feldmann

**Abstract Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, have serious gastrointestinal side effects. Since their direct cytotoxicity was suggested to be involved in this side effect, we here tried to identify NSAID-resistant genes. We screened for *Saccharomyces cerevisiae* genes whose overexpression causes indomethacin resistance and identified the *TPO1* gene, which encodes a major facilitator superfamily transporter. Its overexpression or deletion made yeast cells resistant or sensitive, respectively, to some NSAIDs. A BLAST search identified the possible human orthologue of Tpo1p, tetracycline transporter-like protein (TETRAN), whose overexpression in cultured human cells caused resistance to some NSAIDs, suggesting that TETRAN is an efflux pump for some NSAIDs.**


*Keywords:* Non-steroidal anti-inflammatory drugs; TPO1; Tetracycline transporter-like protein

## 1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most frequently used medicines in the world and account for nearly 5% of all prescribed medications [1]. However, NSAIDs frequently have side effects, including gastrointestinal ulcers and bleeding [2]. Individual variation of sensitivity is a serious clinical problem, but the genetic factors determining this variation are unknown.

Inhibition of cyclooxygenase (COX) by NSAIDs, which is responsible for their anti-inflammatory activity, was previously thought to be fully responsible for their gastrointestinal side effects [3]. However, the increased incidence of gastrointestinal ulcers and the decrease in prostaglandin (PG) levels induced by NSAIDs are not always linked, suggesting that additional mechanisms could be implicated [4]. We recently showed that these involve the direct cytotoxic activity of NSAIDs that is independent on their COX-inhibition activity [5]. Therefore, genes that affect this cytotoxicity may determine risk of gastric lesions, but such genes have not been identified.

For identifying genes that affect the cytotoxicity, we screened *Saccharomyces cerevisiae* genes whose overexpression confers resistance to indomethacin, and we thus identified *TPO1*, which belongs to the major facilitator superfamily (MFS) of transporters. By a BLAST search, we identified a possible human orthologue, tetracycline transporter-like protein (TETRAN), which is predicted to be a drug transporter because of its strong amino acid sequence similarity to a tetracycline transporter in *E. coli* [6]. When TETRAN was overexpressed in cultured human cells, cells became resistant to some NSAIDs. We therefore consider that TETRAN is probably responsible for the efflux of NSAIDs from cells.

## 2. Materials and methods

### 2.1. Plasmids and yeast strains

The *tpo* mutants and the parent strain (YPH499) were kindly donated by Dr. K. Igarashi (Chiba University) [7].

A plasmid containing cDNA for the human *TETRAN* gene was obtained from Invitrogen. After digestion by *Eco*RI and *Xho*I, the resultant DNA fragment was inserted into the *Eco*RI–*Xho*I site of pcDNA3.1(+) to obtain the expression plasmid for TETRAN. Transfection of the plasmid was carried out using Lipofectamine (TM2000) or HilyMax according to the manufacturer's protocol. Transfection of siRNA was performed by HiPerFect transfection reagent according to the manufacturer's instructions. Real-time RT-PCR analysis was done as described [8].

### 2.2. Preparation of yeast genomic library and screening of indomethacin-resistant transformants

Total chromosomal DNA from yeast W303-1 cells was partially digested by *Sau*3AI. DNA fragments (4–10 kb) were purified by ultra-centrifugation in the presence of CsCl, and ligated into the *Bam*HI site of pYES2 (Invitrogen). The resultant yeast genomic library was introduced into W303-1 cells and indomethacin-resistant transformants were selected on synthetic complete (SC) agar plates containing 0.6 mM indomethacin.

### 2.3. Northern blotting

Total RNA was extracted from yeast or human cells by use of an RNeasy kit, according to the manufacturer's specifications. Samples were separated by agarose gel electrophoresis in the presence of 6.3%

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*Abbreviations:* ABC, ATP binding cassette; COX, cyclooxygenase; Drtet, *D. melanogaster* tetracycline resistance protein; FBS, fetal bovine serum; MFS, major facilitator superfamily; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NSAID, non-steroidal anti-inflammatory drug; OD, optical density; PG, prostaglandin; SC, synthetic complete; SNP, single nucleotide polymorphism; TETRAN, tetracycline transporter-like protein

0014-5793/$32.00 
doi:10.1016/j.febslet.2007.03.001

formaldehyde, and blotted onto nylon membranes (Amersham Bioscience). Partial DNA fragments of *TPO1* were amplified by PCR as described [9] and partial DNA fragments of *TETRAN* were produced by digesting the full-length cDNA with *Nde*I and *Aor*51HI. These were radioactively labeled and used as probes.

### 2.4. Assay for sensitivity of cells to NSAIDs

Cells were cultured in RPMI1640 medium containing 10% fetal bovine serum. Cell viability as determined by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method [10]. Apoptotic chromatin condensation was observed as described [10].

### 2.5. Statistical analysis

Values are expressed as a means ± standard error (S.E.M.). One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison was used to evaluate differences between groups. Results were considered to be significant for values of $P < 0.05$.

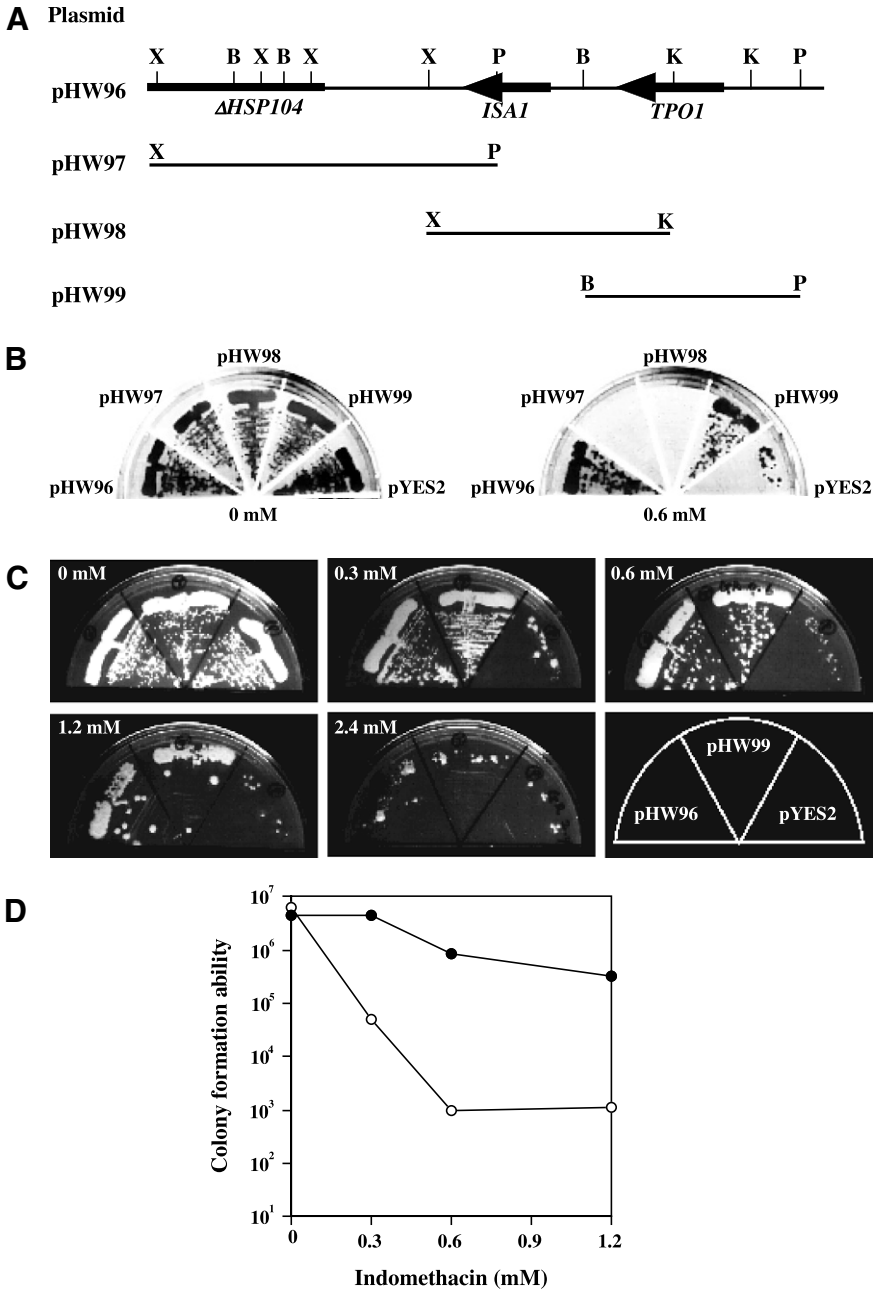


Fig. 1. Identification in yeast of the *TPO1* gene, which caused resistance to indomethacin. (A) Restriction enzyme maps for pHW96 and its derivatives (X, *Xba*I; B, *Bal*I; P, *Pvu*II; K, *Kpn*I). The *GAL* promoter of pYES2 is located on the right side of each insert. (B) W303-1 cells harboring each plasmid were streaked on SC agar plates containing 0 or 0.6 mM indomethacin and plates were incubated at 30 °C for 4 days. (C) W303-1 cells harboring pHW96, pHW99 or the vector (pYES2) were streaked on SC agar plates containing the indicated concentrations of indomethacin, and plates were incubated for 4 days. (D) A full growth suspension of W303-1 harboring pHW99 (closed circle) or pYES2 (open circle) was diluted appropriately and streaked on SC agar plates containing the indicated concentrations of indomethacin. Plates were incubated for 4 days. Colonies were counted and colony-forming units (colonies formed per 1 ml full growth suspension) were determined.

## 3. Results

### 3.1. Screening for indomethacin-resistance genes in yeast

DNA fragments prepared from chromosomes of *S. cerevisiae* W303-1 cells were introduced into pYES2, under the control of the *GAL* promoter. After transformation of W303-1 cells with this DNA library, we identified 18 independent indomethacin resistant colonies from about 20000 transformants on SC agar plates containing 0.6 mM indomethacin. A plasmid from one of clones that were positive after re-transformation experiments (17 clones) was subjected to direct DNA sequencing. The plasmid (pHW96) has two complete genes, *TPO1* and *ISA1*, and a fragment of *HSP104* (Fig. 1A). Subcloning revealed that *TPO1* is responsible for resistance; a plasmid, which contained only *TPO1* (pHW99) made cells resistant to indomethacin and deletion of *TPO1* from pHW96 (pHW97 and pHW98) diminished the resistance (Fig. 1B). Plasmid from other 16 positive clones also contained *TPO1*.

Further analysis was preformed using cells overexpressing *TPO1*. While W303-1/pYES2 did not significantly grow on SC agar plates containing 0.3 mM indomethacin, W303-1/pHW96 and W303-1/pHW99 could grow in the presence of 1.2 mM indomethacin (Fig. 1C). We also found that transformation of W303-1 with pHW99 dramatically increased its ability to form colonies in the presence of 0.3 – 1.2 mM indomethacin (Fig. 1D). Furthermore, we draw growth curve in the presence of various concentrations of indomethacin. While W303-1/pYES2 could not grow in liquid medium containing 0.3 mM indomethacin, W303-1/pHW99 could grow in the presence of 1.2 mM indomethacin (Fig. 2). These results indicate that cells harboring pHW99 are resistant to indomethacin.

W303-1/pYES2 did not grow in liquid medium containing 0.1 mM diclofenac, but W303-1/pHW99 could grow in liquid medium containing 0.2 mM diclofenac. In the presence of ibuprofen, a little difference in growth was also seen, suggesting that the resistance is not specific for indomethacin. On the other hand, in the presence of aspirin, there was no clear difference in growth, showing that W303-1/pHW99 is not resistant to all NSAIDs (Fig. 2).

### 3.2. Sensitivity of tpo mutants to indomethacin

Results described above suggest that Tpo1p protects yeast cells from NSAIDs. To further test this hypothesis, we examined a *tpo1* disruption mutant, Δ*TPO1*. As shown in Fig. 3, the wild-type strain could grow in liquid medium containing 0.2 mM indomethacin, but Δ*TPO*1 mutant did not grow in liquid medium containing 0.1 mM indomethacin, again showing that Tpo1p is involved in indomethacin-resistance in wild-type yeast cells.

Recently, homologues of *TPO1* (*TPO2*, *TPO3* and *TPO4*) were identified in *S. cerevisiae*, all involved in polyamine transport and resistance to polyamines [7]. We examined their contribution to indomethacin resistance, by use of disruption mutants (Δ*TPO*2, Δ*TPO*3 and Δ*TPO*4). As shown in Fig. 3, all mutants (Δ*TPO*2, Δ*TPO*3 and Δ*TPO*4) showed indomethacin-sensitivity similar to that of the wild-type strain. This suggests that indomethacin resistance specifically involves Tpo1p, whereas polyamine resistance involves several genes.

### 3.3. Induction of TPO1 mRNA by indomethacin

Since herbicides induce the transcription of *TPO1* [9], we tested by Northern blotting analysis whether indomethacin also induces the transcription of *TPO1*. As shown in Fig. 4,

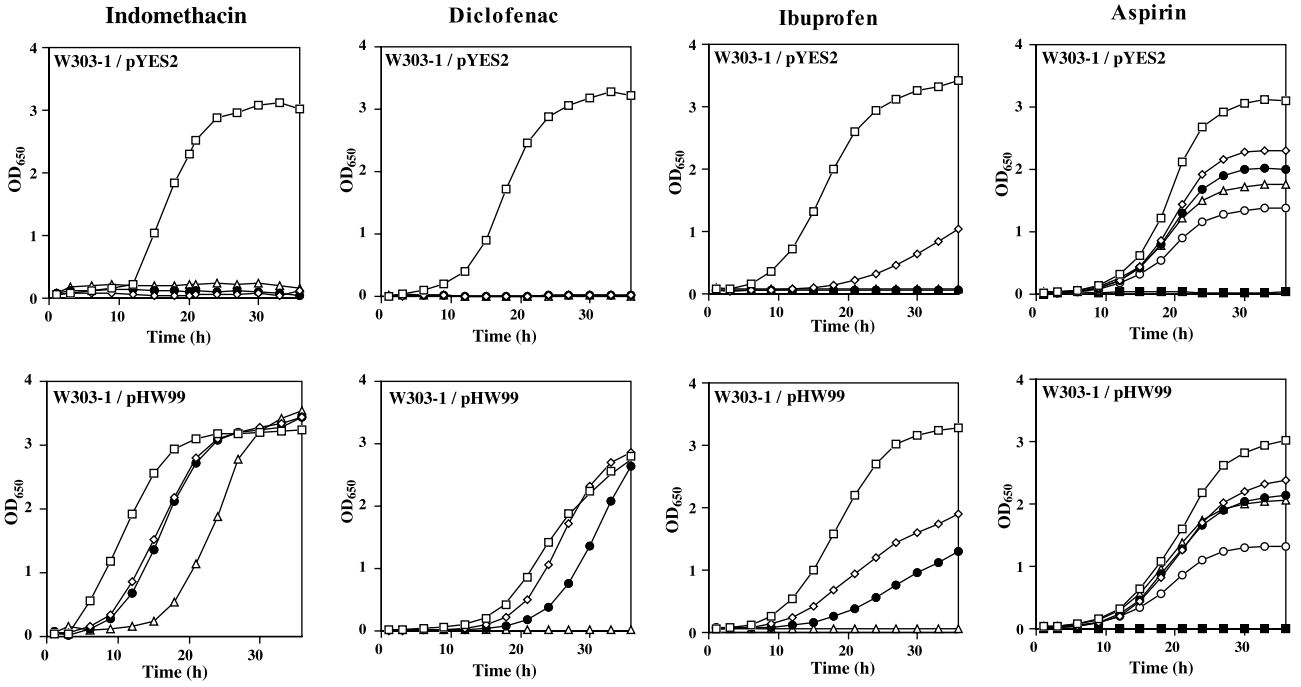


Fig. 2. Growth of yeast cells overexpressing *TPO1* in the presence of NSAIDs. Full growth suspensions of W303-1/pHW99 or W303-1/pYES2 were 1/50 diluted and cultured at 30 °C in the presence of 0 (open square), 0.3 (open diamond), 0.6 (closed circle), and 1.2 (open triangle) mM indomethacin; 0 (open square), 0.1 (open diamond), 0.2 (closed circle), and 0.4 (open triangle) mM diclofenac; 0 (open square), 0.1 (open diamond), 0.2 (closed circle), and 0.4 (open triangle) mM ibuprofen; 0 (open square), 1 (open diamond), 2 (closed circle), 4 (open triangle), 8 (open circle), and 10 (closed square) mM aspirin. The optical density (OD) at 650 nm was monitored.

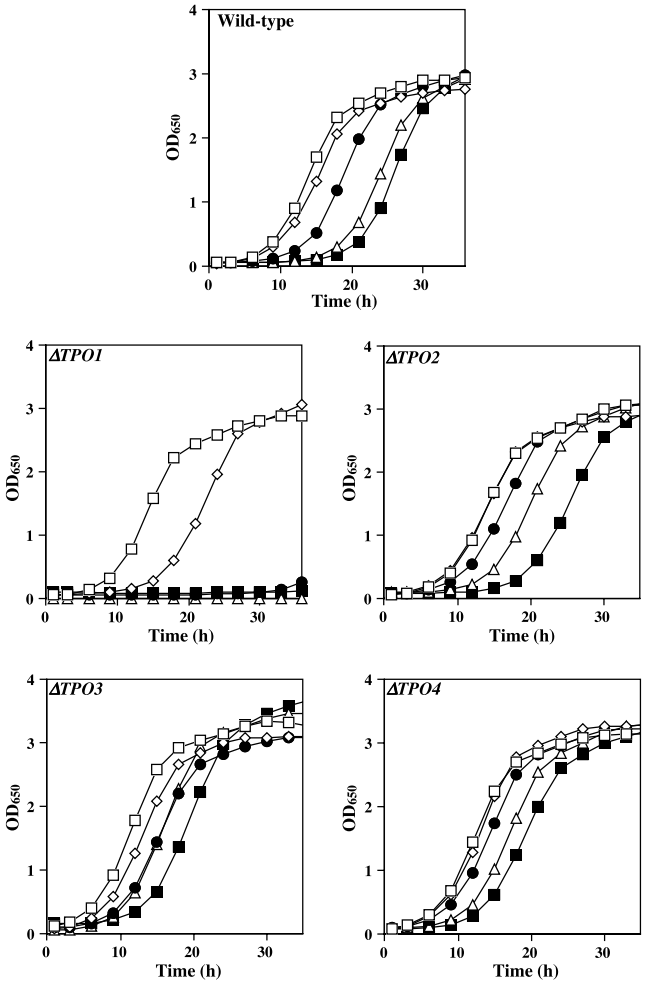


Fig. 3. Growth of yeast *tpo* mutants in the presence of indomethacin. Full growth suspensions of Δ*TPO*1, Δ*TPO*2, Δ*TPO*3, Δ*TPO*4 and the wild-type strain, YPH499, were 1/50 diluted and cultured at 30 °C in the presence of 0 (open square), 0.05 (open diamond), 0.1 (closed circle), 0.15 (open triangle) and 0.2 (closed square) mM indomethacin. The OD at 650 nm was monitored.

*TPO1* mRNA was induced by indomethacin. The induction of *TPO1* probably contributed to the protection of yeast cells from indomethacin.

### 3.4. Identification of the human orthologue of TPO1

A direct BLAST search of the human genome, using the amino acid sequence of yeast Tpo1p as a query, did not find any matching genes. We therefore searched for the *D. melanogaster* orthologue of Tpo1p in a similar way and identified the tetracycline resistance protein (Drtet; AE003733 [GenBank]), which is predicted to be tetracycline efflux pump, based on its amino acid sequence (information from GenBank homepage). Drtet shows 8% identity and 25% similarity to yeast Tpo1p in total. Finally, we identified a human orthologue of Drtet, TETRAN (L11669 [GenBank]) [6]. TETRAN shows 40% identity and 74% similarity to Drtet in total. TETRAN has putative 12-membrane-spanning domains, belongs to the MFS class of proteins, and has significant similarity to the *E. coli* tetracycline transporter (TetA) [6], which has also 12-membrane-spanning domains and also belong to MFS [11]. As shown in Fig. 5, some amino acid residues were conserved

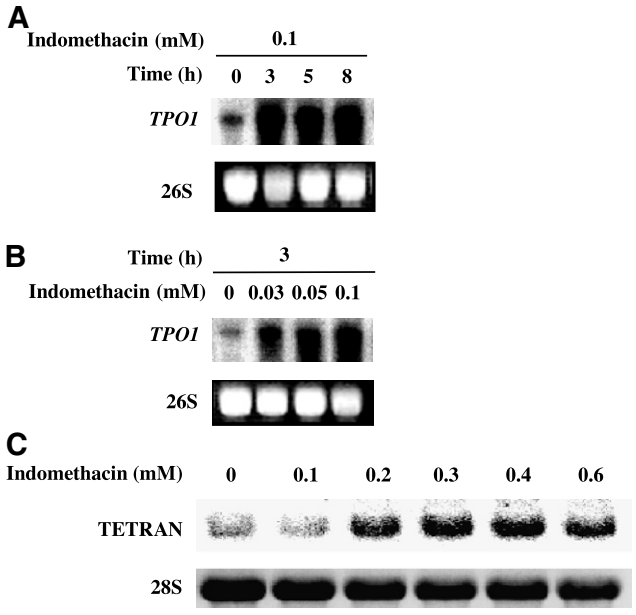


Fig. 4. Induction of *TPO1* mRNA by indomethacin. Exponentially growing W303-1 cells were (A) treated with 0.1 mM indomethacin for the indicated periods or (B) with the indicated concentrations of indomethacin for 3 h. MKN45 ells were exposed to various concentrations of indomethacin for 24 h (C). The level of *TPO1* mRNA (A, B) or *TETRAN* mRNA (C) was monitored by northern blotting analysis. Lower panel shows ribosomal RNA (26S or 28S) stained with ethidium bromide.

between TETRAN, Drtet and Tpo1p. These data suggest that TETRAN is the human orthologue of Tpo1p.

### 3.5. Effect of TETRAN on the sensitivity of cultured human cells to NSAIDs

No biochemical and biological activities have been reported for TETRAN. To characterize TETRAN, we obtained stable transfectants of MKN45 (a human adenocarcinoma gastric cell line) with an expression plasmid containing *TETRAN* gene. This clone expressed about 2 times more TETRAN mRNA than the control strains. This TETRAN-overexpressing clone and control clone were incubated with various concentrations of indomethacin and the cell viability was determined. As shown in Fig. 6A, the TETRAN-overexpressing clone was more resistant to indomethacin than vector-transfected cells. Furthermore, the TETRAN-overexpressing clone was also resistant to diclofenac (Fig. 6B), suggesting that overexpression of TETRAN in MKN45 cells makes cells resistant to some NSAIDs.

Based on our previous report [12], we considered that cell death seen in Fig. 6A and B is mediated by apoptosis, in other words, overexpression of TETRAN makes cells resistant to NSAID-induced apoptosis. For confirming this point, we examined the effect of overexpression of TETRAN in MKN45 cells on NSAID-induced apoptosis by counting cells with apoptotic chromatin condensation. As shown in Fig. 6C, treatment with indomethacin caused less apoptotic cells in TETRAN-overexpressing clone than in control clone (mock). Similar results were obtained with diclofenac (Fig. 6C), suggesting that overexpression of TETRAN makes cells resistant to NSAID-induced apoptosis. For further confirmation of this point, we used the siRNA for TETRAN.

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TETRAN        1 ----------------------------------------------------MGWGGG 6
Drtet         1 ----------------------------------------------------MADLRSRHN 9
Tpo1          1 MSDHSPISNKENHLLPSDSSRSSSSDMHSTGTTGTTGVEPVDFTGEGAKYTTATEGNGGA 60
Homology

TETRAN        7 GGCTPRPPIHQQP--------------------PERR--VVIVVFLGLLLD--------- 35
Drtet        10 GTAAMEKQSHSQTGSHHHNNNKALDKEASENGKPEKSDPMIYIIFVSLLFD--------- 60
Tpo1         61 DLAIQRTTTMNSAAESEVNITRRLTKILTGSVNEPDRVEVDYTNCAPMGGDRPYPPSLPS 120
Homology        . .  .    ...                            :       :  *

TETRAN       36 --LLAFTLLLP---LLPGLLESHGRAHDP-LYGSWQGGVDWFATAIGMPV---EKRYNSV 86
Drtet        61 --LLAFTIILP---LLPSLLEHYRQNDSSGLYAVLTDRVRWFQQLLGAP-----DRYISV 110
Tpo1        121 RDLYEVTFDGPNDPLHPFNWPMKKKVLLCLVLCLDSIAIAMCSSIFASAVPQICEIYHVI 180
Homology          *  .*:  *   * *       :     :       :      :. .     . *  :

TETRAN       87 LFGGLIGSAFSVLQFLCAPLTGA-TSDCLGRRPVMLLCLMGVATSYAVWATSRSFAAFLA 145
Drtet       111 LFGGFLGSMFSFLQFVASPIVGG-LSDYYGRKPVLLACASGIALSYLIWACSSNFALFVL 169
Tpo1        181 EVVAILGITLFVLGFAASPVIYAPLSELYGRKGVLVLSAFGFALFQFAVATAENLQTIFI 240
Homology         . .::*  : .* * .:*:  .  *:  **: *:: .  *.*      * : .:  :.

TETRAN      146 SRLIGGIS-KGNVSLSTAIVADLGSPLARSQGMAVIGVAFSLGFTLGPMLGASLPLEMAP 204
Drtet       170 ARFVGGIS-KGNISLCMSVITDVSSVKTRGRGMALVGVAFSLGFIVGPMIGALFAIFSDK 228
Tpo1        241 CRFFGGFIGAAPMAVVPAAFADMFDTNVRGKAIALFSLGVFVGPILSPVMGSYIAQRTTW 300
Homology        .*:.**:   . :::  : .:*: .  .*.:.:*:..:.. :*  :.*::*: :.

TETRAN      205 ----------WFALLFAASDLLFIFCFLPETLP--------LEKR-------APSIALGF 239
Drtet       229 SGSTWFVLPSLLAFGLAVGDLVVLACCLRETLP--------KEKR-------VKEISSAL 273
Tpo1        301 R------WLEYVVGCFASAVFVAIVLFFEETHHPTILVNKAKQMRKQSNNWGIHAAHEDV 354
Homology                 ..  :* . :: :   : **           : *             .

TETRAN      240 RDAADLLSPLALLRFSAVARGQDPPSGDRLSSLRRLGLVYFLYLFLFSGLEYTLSFLTHQ 299
Drtet       274 SYGLQLLNFSAIFRFAAIKNVPK----KDIAALRSIGLVYFLYLFLYSGLEFTVTFLMYH 329
Tpo1        355 ELSIKDIVQKTVTRPIIMLFVEPLLLFVTIYNSFVYGILYLLLEAYPLVFVEGYGFTENG 414
Homology          . . :   :: *   :           :      *::*:*       :     *

TETRAN      300 RFQFSSLQQGKMFFLIGLTMATIQGAYARRIHPGGEVAAVKRALLLLVPAFLLIGWGRSL 359
Drtet       330 KFGYTSMDQAKMFLTTGVIMTLLQGSVVRRLPEAKIKGYAIFSLYLIVPAFVVVGLAEGS 389
Tpo1        415 ELPYIALIIGMMVCAAFIWYMDNDYLKRCRAKGKLVPEARLYAMVIAGTVFPIGILWFCW 474
Homology        .: : ::  . *.    :     :     *            :: :  ..* :

TETRAN      360 PVLG-----------LGLLLYSFAAAVVVPCLSSVVAGYGSPGQKGTVMGTLR--SLGAL 406
Drtet       390 RMLY-----------AGMTLFAISTAFAVTCLTTLVSKYGNDDQKGSVLGIFR--SLGAL 436
Tpo1        475 TGYYPHKIHWMVPTVGGAFIGFGLMGIFLPCLNYIIESYLLLAASAVAANTFMRSAFGAC 534
Homology                      *  :     .. :.**. ::  *     .. . . :   ::**

TETRAN      407 ARAAG--PLVAASVYWLAGAQACFTTWSGLFLLPFFLLQKLSYPAQTLKAE-         455
Drtet       437 ARALG--PVVGCIAFWCVGSRITYIAGGLLLIYPAMALQRARI---------         477
Tpo1        535 FPLFAGYMFRGMGIGWAGLLLGLFAAAMIPVPLLFLKYGESIRKKSKYAYAA         586
Homology            .   . .    *       : :    .    :   .
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Fig. 5. Amino acid sequences of human TETRAN, *D. melanogaster* Drtet, and *S. cerevisiae* TPO1. The alignment was performed by CLUSTAL W from the Pole Bio-Informatique Lyonnais web server (* identical residues, : strongly similar residues, · weakly similar residues).

As shown in Fig. 7A, transfection of siRNA for TETRAN into MKN45 cells caused suppression of the mRNA expression of TETRAN in its dose-dependent manner. Transfection of this siRNA stimulated the apoptosis induced by indomethacin or diclofenac (Fig. 7B), confirming that expression of TETRAN makes cells resistant to NSAID-induced apoptosis. The trasnfection of this siRNA also elevated the background level of apoptosis (without NSAIDs), suggesting that expression of TETRAN also suppresses the spontaneous apoptosis under the conditions.

We also examined the effect of indomethacin on the expression of *TETRAN* mRNA in MKN45 cells. As shown in

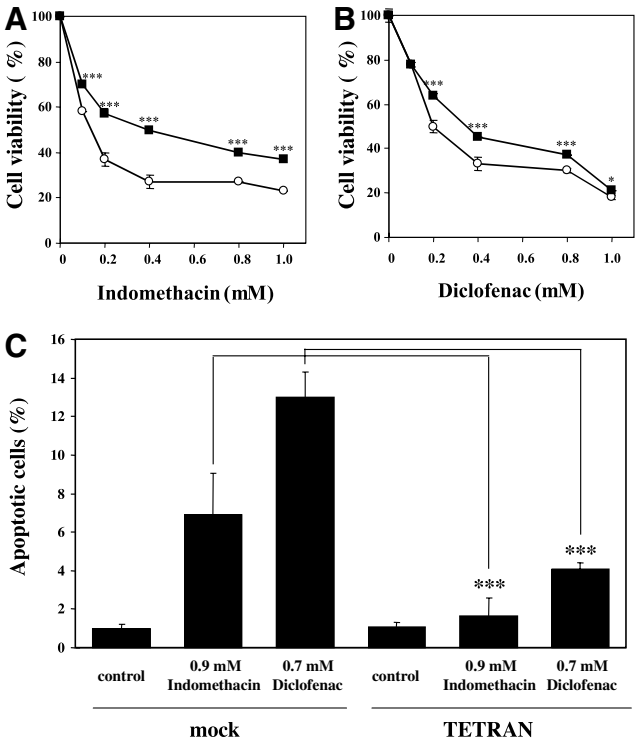


Fig. 6. Sensitivity to NSAIDs of human cells overexpressing TETRAN. A stable transfectant of gastric carcinoma line MKN45 with the expression vector for TETRAN (closed square) and with the vector only (open circle) were exposed for 38 h to the indicated concentrations of indomethacin or diclofenac. Cell viability was determined by the MTT method (A, B). Apoptotic cells with condensed chromatins were counted and expressed relative to total cells (C).Values are means ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{***}P < 0.001$.

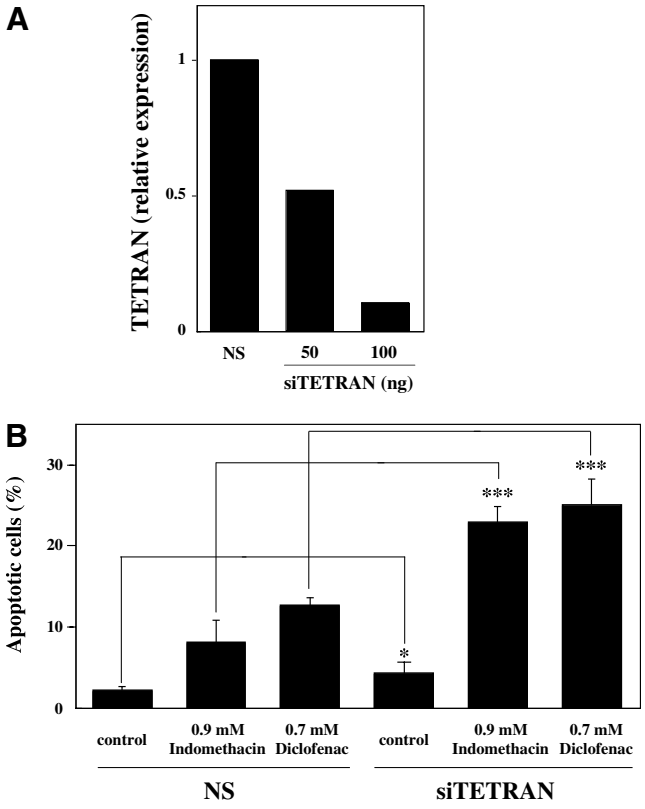


Fig. 7. Effect of siRNA for TETRAN on NSAIDs-induced apoptosis. MKN45 cells were transfected with indicated amounts (A) or 100 ng (B) of siRNA for TETRAN (siTETRAN) or non-silencing siRNA (NS) and cultured for 24 h. Cells were further incubated with or without indicated concentration of indomethacin or diclofenac for 24 h (B). The levels of *TETRAN* mRNA were estimated by real-time RT-PCR experiments using a specific primer for each gene. Values were normalized to actin gene expression and expressed relative to the control sample (A). Apoptosis was monitored as described in the legend of Fig. 6 (B). Values are means ± S.E.M. ($n = 3$). $^{*}P < 0.05$, $^{***}P < 0.001$.

Fig. 4C, indomethacin induced the expression of *TETRAN* mRNA in a dose-dependent manner. This induction probably contributes to the protection of gastric cells from indomethacin.

## 4. Discussion

Patients vary in sensitivity to the gastrointestinal side effects of NSAIDs, making clinical use difficult. As the first step to understand this variation, we tried to identify genes that affect the direct cytotoxicity of NSAIDs, which may be at least partly responsible for causing gastric ulcers [5]. In yeast, the *TPO1* gene was shown to be involved in NSAID-resistance. It is the first such gene to be identified in yeast. We also identified a possible human orthologue, TETRAN. Overexpression of TETRAN or suppression of its expression by siRNA technique made cultured human cells resistant or sensitive, respectively, to some NSAIDs. TETRAN is the first protein shown to affect indomethacin resistance in human cells. In future, single nucleotide polymorphism (SNP) analysis of TETRAN may be important to understand the mechanism of variation of patients for sensitivity to the gastrointestinal side effects of NSAIDs.

Tpo1p has putative12 membrane-spanning domains, belongs to the MFS class of proteins, and is located on plasma membranes [13,14]. It was first identified as a transporter for polyamines [7,13], however, at present, it is thought to be a multidrug efflux pump, because its expression also caused resistance to herbicides, cycloheximide, quinidine and immunosuppressive drugs [9,15–17]. Therefore, Tpo1p is probably involved in indomethacin export from yeast cells.

Based on the sequence similarity between TETRAN and other drug efflux pumps, including Tpo1p, we consider that TETRAN is probably an efflux pump for NSAIDs in human cells. TETRAN's amino acid sequence strongly suggests that it also belongs to the MFS class of proteins [6]. Drug efflux pumps can be separated into two groups based on the mode of transport and energy source: primary and secondary active transporters. Primary active transporters are also referred to as ATP binding cassette (ABC) proteins, and use the energy of ATP hydrolysis. Secondary active transporters, e.g. MFS class proteins, act as anti-ports coupled with ion transport [18]. In bacteria, secondary active transporters are predominant for drug efflux [19], but in eukaryotic cells, primary active transporters are predominant [20]. TETRAN is the first MFS protein identified that is involved in drug resistance in human cells, and further studies of such proteins will be important to understand drug efflux mechanisms in human cells.

*Acknowledgments:* We thank Drs. K. Igarashi and K. Kashiwagi (Chiba University) for giving us the *tpo* mutants and for helpful discussion.

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